

HAEMOPHILUS ADHERENCE AND PENETRATION PROTEINS

This is a continuation-in-part application of application serial number 08/296,791 filed October 25, 1994, now U.S. Patent No. 6,245,337 and serial number 09/839,996, filed April 20, 2001, pending.

FIELD OF THE INVENTION

- 5 The invention relates to *Haemophilus* adhesion and penetration proteins, nucleic acids, and vaccines.

BACKGROUND OF THE INVENTION

10 Most bacterial diseases begin with colonization of a particular mucosal surface (Beachey et al., 1981, J. Infect. Dis. 143:325-345). Successful colonization requires that an organism overcome mechanical cleansing of the mucosal surface and evade the local immune response. The process of colonization is dependent upon specialized microbial factors that promote binding to host cells (Hultgren et al., 1993 Cell, 73:887-901). In some cases the colonizing organism will subsequently enter (invade) these cells and survive intracellularly (Falkow, 1991, Cell 65:1099-1102).

15 *Haemophilus influenzae* is a common commensal organism of the human respiratory tract (Kuklinska and Kilian, 1984, Eur. J. Clin. Microbiol. 3:249-252). It is a human-specific organism that normally resides in the human nasopharynx and must colonize this site in order to avoid extinction. This microbe has a number of surface structures capable of promoting attachment to host cells (Guerina et al., 1982, J. Infect. Dis. 146:564; Pichichero et al., 1982, Lancet ii:960-962; St. Geme et al., 1993, Proc. Natl. Acad. Sci. U.S.A. 90:2875-2879). In addition, *H. influenzae* has acquired the capacity to enter and survive within these cells (Forsgren et al., 1994, Infect. Immun. 62:673-679; St. Geme and Falkow, 1990, Infect. Immun. 58:4036-4044; St. Geme and Falkow, 1991, Infect. Immun. 59:1325-1333, Infect. Immun. 59:3366-3371). As a result, this bacterium is an important cause of both localized respiratory tract and systemic disease (Turk, 1984, J. Med. Microbiol. 18:1-16). Nonencapsulated, non-
20 typable (NT) strains account for the majority of local disease (Turk, 1984, supra); in contrast, serotype b strains, which express a capsule composed of a polymer of ribose and ribitol-5-phosphate (PRP), are responsible for over 95% of cases of *H. influenzae* systemic disease (Turk, 1982, Clinical importance of *Haemophilus influenzae*, p. 3-9. In S.H. Sell and P.F. Wright (ed.), *Haemophilus influenzae* epidemiology, immunology, and prevention of disease. Elsevier/North-Holland Publishing Co., New York).

25 The initial step in the pathogenesis of disease due to *H. influenzae* involves colonization of the upper respiratory mucosa (Murphy et al., 1987, J. Infect. Dis. 5:723-731). Colonization with a particular strain may persist for weeks to months, and most individuals remain asymptomatic throughout this period (Spinosa et al., 1986, I. Infect. Dis. 154:100-109). However, in certain
35 circumstances colonization will be followed by contiguous spread within the respiratory tract, resulting in local disease in the middle ear, the sinuses, the conjunctiva, or the lungs.

Alternatively, on occasion bacteria will penetrate the nasopharyngeal epithelial barrier and enter the bloodstream.

5 *In vitro* observations and animal studies suggest that bacterial surface appendages called pili (or fimbriae) play an important role in *H. influenzae* colonization. In 1982 two groups reported a correlation between piliation and increased attachment to human oropharyngeal epithelial cells and erythrocytes (Guerina *et al.*, supra; Pichichero *et al.*, supra). Other investigators have demonstrated that anti-pilus antibodies block *in vitro* attachment by piliated *H. influenzae* (Forney *et al.*, 1992, J. Infect. Dis. 165:464-470; van Alphen *et al.*, 1988, Infect. Immun. 56:1800-1806). Recently Weber *et al.* insertionally inactivated the pilus structural gene in an
10 *H. influenzae* type b strain and thereby eliminated expression of pili; the resulting mutant exhibited a reduced capacity for colonization of year-old monkeys (Weber *et al.*, 1991, Infect. Immun. 59:4724-4728).

A number of reports suggest that nonpilus factors also facilitate *Haemophilus* colonization. Using the human nasopharyngeal organ culture model, Farley *et al.* (1986, J. Infect. Dis. 161:274-280) and Loeb *et al.* (1988, Infect. Immun. 49:484-489) noted that nonpiliated type b
15 strains were capable of mucosal attachment. Read and coworkers made similar observations upon examining nontypable strains in a model that employs nasal turbinate tissue in organ culture (1991, J. Infect. Dis. 163:549-558). In the monkey colonization study by Weber *et al.* (1991, supra), nonpiliated organisms retained a capacity for colonization, though at reduced
20 densities; moreover, among monkeys originally infected with the piliated strain, virtually all organisms recovered from the nasopharynx were nonpiliated. All of these observations are consistent with the finding that nasopharyngeal isolates from children colonized with *H. influenzae* are frequently nonpiliated (Mason *et al.*, 1985, Infect. Immun. 49:98-103; Brinton *et al.*, 1989, Pediatr. Infect. Dis. J. 8:554-561).

25 Previous studies have shown that *H. influenzae* are capable of entering (invading) cultured human epithelial cells via a pili-independent mechanism (St. Geme and Falkow, 1990, supra; St. Geme and Falkow, 1991, supra). Although *H. influenzae* is not generally considered an intracellular parasite, a recent report suggests that these *in vitro* findings may have an *in vivo* correlate (Forsgren *et al.*, 1994, supra). Forsgren and coworkers examined adenoids from
30 children who had their adenoids removed because of longstanding secretory otitis media or adenoidal hypertrophy. In all 10 cases there were viable intracellular *H. influenzae*. Electron microscopy demonstrated that these organisms were concentrated in the reticular crypt epithelium and in macrophage-like cells in the subepithelial layer of tissue. One possibility is that bacterial entry into host cells provides a mechanism for evasion of the local immune
35 response, thereby allowing persistence in the respiratory tract.

Thus, a vaccine for the therapeutic and prophylactic treatment of *Haemophilus* infection is desirable. Accordingly, it is an object of the present invention to provide for recombinant *Haemophilus* Adherence and Penetration (HAP) proteins and variants thereof, and to produce useful quantities of these HAP proteins using recombinant DNA techniques.

40 It is a further object of the invention to provide recombinant nucleic acids encoding HAP proteins, and expression vectors and host cells containing the nucleic acid encoding the HAP protein.

An additional object of the invention is to provide monoclonal antibodies for the diagnosis of *Haemophilus* infection.

A further object of the invention is to provide methods for producing the HAP proteins, and a vaccine comprising the HAP proteins of the present invention. Methods for the therapeutic and prophylactic treatment of *Haemophilus* infection are also provided.

SUMMARY OF THE INVENTION

In accordance with the foregoing objects, the present invention provides recombinant HAP proteins, and isolated or recombinant nucleic acids which encode the HAP proteins of the present invention. Also provided are expression vectors which comprise DNA encoding a HAP protein operably linked to transcriptional and translational regulatory DNA, and host cells which contain the expression vectors.

The invention also provides methods for producing HAP proteins which comprises culturing a host cell transformed with an expression vector and causing expression of the nucleic acid encoding the HAP protein to produce a recombinant HAP protein.

The invention also includes vaccines for *Haemophilus influenzae* infection comprising an HAP protein for prophylactic or therapeutic use in generating an immune response in a patient. Methods of treating or preventing *Haemophilus influenzae* infection comprise administering a vaccine.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B depict light micrographs of *H. influenzae* strains DB117(pGJB103) and DB117(pN187) incubated with Chang epithelial cells. Bacteria were incubated with an epithelial monolayer for 30 minutes before rinsing and straining with Giemsa stain. Figure 1A: *H. influenzae* strain DB117 carrying cloning vector alone (pGJB103); Figure 1B: *H. influenzae* strain DB117 harboring recombinant plasmid pN187. Bar represents 3.5 μ m.

Figures 2A, 2B, 2C and 2D depict thin section transmission electron micrographs demonstrating interaction between *H. influenzae* strains N187 and DB117(pN187) with Chang epithelial cells. Bacteria were incubated with epithelial monolayers for four hours before rinsing and processing for examination by transmission electron microscopy. Figure 2A: strain N187 associated with the epithelial cell surface and present in an intracellular location; Figure 2B: *H. influenzae* DB117 (pN187) in intimate contact with the epithelial cell surface; Figure 2C: strain DB117(pN187) in the process of entering an epithelial cell; Figure 2D: strain DB117(pN187) present in an intracellular location. Bar represents 1 μ m.

Figure 3 depicts outer membrane protein profiles of various strains. Outer membrane proteins were isolated on the basis of sarcosyl insolubility and resolved on a 10% SDS-polyacrylamide gel. Proteins were visualized by staining with Coomassie blue. Lane 1, *H. influenzae* strain DB117(pGJB103); lane 2, strain DB117(pN187); lane 3, strain DB117(pJS106); lane 4, *E. coli* HB101(pGJB103); lane 5, HB101(pN187). Note novel proteins at ~160 kD and 45 kD marked by asterisks in lanes 2 and 3.

Figure 4 depicts a restriction map of pN187 and derivatives and locations of mini-Tn10 *kan* insertions. pN187 is a derivative of pGJB103 that contains an 8.5-kb *Sau3AI* fragment of chromosomal DNA from *H. influenzae* strain N187. Vector sequences are represented by hatched boxes. Letters above top horizontal line indicate restriction enzyme sites: Bg, *Bgl*II; C, *Clal*; E, *EcoRI*; P, *Pst*I. Numbers and lollipops above top horizontal line show positions of mini-Tn10 *kan* insertions; open lollipops represent insertions that have no effect on adherence and invasion, while closed lollipops indicate insertions that eliminate the capacity of pN187 to promote association with epithelial monolayers. Heavy horizontal line with arrow represents location of *hap* locus within pN187 and direction of transcription. (+): recombinant plasmids that promote adherence and invasion; (-): recombinant plasmids that fail to promote adherence and invasion.

Figure 5 depicts the identification of plasmid-encoded proteins using the bacteriophage T7 expression system. Bacteria were radiolabeled with [³⁵S] methionine, and whole cell lysates were resolved on a 10% SDS-polyacrylamide gel. Proteins were visualized by autoradiography. Lane 1, *E. coli* XL-1 Blue(pT7-7) uninduced; lane 2, XL-1 Blue(pT7-7) induced with IPTG; lane 3, XL-1 Blue(pJS103) uninduced; lane 4, XL-1 Blue(pJS103) induced with IPTG; lane 5, XL-1 Blue(pJS104) uninduced; lane 6, XL-1 Blue(pJS104) induced with IPTG. The plasmids pJS103 and pJS104 are derivatives of pT7-7 that contain the 6.7-kb *Pst*I fragment from pN187 in opposite orientations. Asterisk indicates overexpressed protein in XL-1 Blue(pJS104).

Figures 6A, 6B, and 6C depict the nucleotide sequence (SEQ ID NO: 1) and predicted amino acid sequence (SEQ ID NO: 2) of *hap* gene. Putative -10 and -35 sequences 5' to the *hap* coding sequence are underlined; a putative *rho*-independent terminator 3' to the *hap* stop codon is indicated with inverted arrows. The first 25 amino acids of the protein, which are boxed, represent the signal sequence.

Figures 7A, 7B, 7C, 7D, 7E, 7F, 7G, and 7H depict a sequence comparison of the *hap* product (SEQ ID NO: 2) and the cloned *H. influenzae* IgA1 proteases (SEQ ID NO: 3-6). Amino acid homologies between the deduced *hap* gene product and the *iga* gene products from *H. influenzae* HK368 (SEQ ID NO: 3), HK61 (SEQ ID NO: 6), HK393 (SEQ ID NO: 4), and HK715 (SEQ ID NO: 5) are shown. Dashes indicate gaps introduced in the sequences in order to obtain maximal homology. A consensus sequence for the five proteins is shown on the lower line. The conserved serine-type protease catalytic domain is underlined, and the common active site serine is denoted by an asterisk. The conserved cysteines are also indicated by asterisks.

Figure 8 depicts the IgA1 protease activity assay. Culture supernatants were assayed for the ability to cleave IgA1. Reaction mixtures were resolved on a 10% SDS-polyacrylamide gel and then transferred to a nitrocellulose membrane. The membrane was probed with antibody against human IgA1 heavy chain. Lane 1, *H. influenzae* strain N187; lane 2, strain DB117(pGJB103); lane 3, strain DB117(pN187). The cleavage product patterns suggest that strain N187 contains a type 2 IgA1 protease while strains DB117(pGJB103) and DB117(pN187) contain a type 1 enzyme. The upper band of ~70-kD seen with the DB117 derivatives represents intact IgA1 heavy chain.

Figures 9A and 9B depict southern analysis of chromosomal DNA from strain *H. influenzae* N187, probing with *hap* versus *iga*. DNA fragments were separated on a 0.7% agarose gel and transferred bidirectionally to nitrocellulose membranes prior to probing with either *hap* or *iga*. Lane 1, N187 chromosomal DNA digested with *EcoRI*; lane 2, N187 chromosomal DNA digested with *BglII*; lane 3, N187 chromosomal DNA digested with *BamHI*; lane 4, the 4.8-kb *Clal-PstI* fragment from pN187 that contains the intact *hap* gene. Figure 9A: Hybridization with the 4.8-kb *Clal-PstI* fragment containing the *hap* gene; Figure 9B: hybridization with the *iga* gene from *H. influenzae* strain Rd, carried as a 4.8-kb *Clal-EcoRI* fragment in pVD116.

Figure 10 depicts a SDS-polyacrylamide gel of secreted proteins. Bacteria were grown to late log phase, and culture supernatants were precipitated with trichloroacetic acid and then resolved on a 10% SDS-polyacrylamide gel. Proteins were visualized by staining with Coomassie blue. Lane 1, *H. influenzae* strain DB117(pGJB103); lane 2, DB117(pN187); lane 3, DB117(pJS106); lane 4, DB117(pJS102); lane 5, DB117(pJS105); lane 6, DB117(Tn10-18); lane 7, DB117(Tn10-4'); lane 8, DB117(Tn10-30); lane 9, DB117(Tn10-16); lane 10, DB117(Tn10-10); lane 11, DB117(Tn10-8); lane 12, N187. Asterisk indicates 110-kD secreted protein encoded by *hap*.

Figure 11 depicts an alignment of the deduced amino acid sequence of HAP proteins obtained from various *H. influenzae* strains. The strains include N187 (SEQ ID NO: 7), TN106 (SEQ ID NO: 11) and 860295 (SEQ ID NO: 13).

Figure 12 depicts Western blot of Hap proteins. Panel A shows outer membrane proteins, and panel B shows culture supernatants after precipitation with trichloroacetic acid. In each panel, lane 1 contains DB117/pGJB103 (vector), lane 2 contains DB117/pJS106 (encoding HapN187), lane 3 contains DB117/pHapP860295, and lane 4 contains DB117/pHapTN106. Immunoblotting was performed with guinea pig antiserum GP74, which was raised against purified Hap_s from strain N187 and recognizes full-length Hap in outer membranes and Hap_s in culture supernatants. Without being bound by theory, it is thought that the lower band in lane 3 of panel B presumably reflects autoproteolysis of multiple sites in Hap from strain P860295 (Fink et al; 2001).

Figure 13 Adherence to Chang epithelial cells by *H. influenzae* strain DB117 expressing HapN187, HapTN106, or HapP860295 and the inhibitory effect of preincubation with anti-Haps antiserum. Adherence was determined in 30 minute assays and was calculated by dividing the number of adherent bacteria by the number of inoculated bacteria. For all strains, inocula were approximately 2×10^7 CFU/ml. Bars represent the mean + standard error of the mean.

Figure 14 depicts SDS-PAGE of purified HAPs proteins from both strain P860295 and strain N187. Amino terminal amino acid sequencing confirmed that purified protein was Haps.

Figure 15 depicts clearance of NTHi TN106.P2 in Balb/c mice vaccinated with Hap_s P860295 with and without CT-E29H. Six week old female Balb/c mice were vaccinated intranasally with Hap_s from P860295, HAP+CT-E29H, or Formalin Fixed TN106.P2 in a 40 μ l volume at weeks 0, 1, 3, & 5. Five animals from each group were IN challenged with $\sim 1 \times 10^6$ CFU/mouse in 10 μ l, 2 and 3 weeks post vaccination. Nasal tissue were harvested 3 days after challenge.

(*=Statistically different from 0.1 µg E29H control/Student's T test; @=Statistically different from PBS control/Student's T test).

Figure 16 depicts the nucleotide sequence for NTHi strain 11 *hap* gene (SEQ ID NO: 8) (start codon to stop codon).

5 Figure 17 depicts the amino acid sequence for NTHi strain 11 Hap protein (SEQ ID NO: 9) (first amino acid to last amino acid).

Figure 18 depicts the nucleotide sequence for NTHi strain TN106 *hap* gene (SEQ ID NO: 10) (start codon begins at position 422, stop codon begins at position 4595).

10 Figure 19 depicts the amino acid sequence for NTHi strain TN106 Hap protein (SEQ ID NO: 11) (first amino acid to last amino acid).

Figure 20 depicts the nucleotide sequence for NTHi strain 860295 *hap* gene (SEQ ID NO: 12) (start codon begins at position 430, stop codon begins at position 4738).

Figure 21 depicts the amino acid sequence for NTHi strain 860295 Hap protein (SEQ ID NO: 13) (first amino acid to last amino acid).

15 Figure 22 depicts the nucleotide sequence for NTHi strain 3219B *hap* gene (SEQ ID NO: 14) (start codon begins at position 388, stop codon begins at position 4561).

Figure 23 depicts the amino acid sequence for NTHi strain 3219B Hap protein (SEQ ID NO: 15) (first amino acid to last amino acid).

20 Figure 24 depicts the nucleotide sequence for NTHi strain 1396B *hap* gene (SEQ ID NO: 16) (start codon begins at position 313, stop codon begins at position 4546).

Figure 25 depicts the amino acid sequence for NTHi strain 1396B Hap protein (SEQ ID NO: 17) (first amino acid to last amino acid).

DETAILED DESCRIPTION OF THE INVENTION

25 The present invention provides novel *Haemophilus* Adhesion and Penetration (HAP) proteins. In a preferred embodiment, the HAP proteins are from *Haemophilus* strains, and in the preferred embodiment, from *Haemophilus influenzae*. However, using the techniques outlined below, HAP proteins from other *Haemophilus influenzae* strains, including but not limited to NTHi TN 106, TN106.P2, N187, P860295, 11, 3219B, 1396B or from other bacterial species such as *Neisseria* spp. or *Bordetella* spp. may also be obtained.

30 A HAP protein may be identified in several ways. A HAP nucleic acid or HAP protein is initially identified by substantial nucleic acid and/or amino acid sequence homology to the sequences shown in Figure 6. Such homology can be based upon the overall nucleic acid or amino acid sequence. In addition a HAP protein is identifiable by substantial amino acid sequence homology or identity to the sequences shown in Figures 11 (SEQ ID NOS 7, 11, 13), 17 (SEQ

ID NO 9), 19 (SEQ ID NO 11), 21 (SEQ ID NO 13), 23 (SEQ ID NO 15) or 25 (SEQ ID NO 17). In addition a HAP nucleic acid is identified by substantial nucleic acid sequence identity to the sequences set forth in Figures 16 (SEQ ID NO 8), 18 (SEQ ID NO 10), 20 (SEQ ID NO 12), 22 (SEQ ID NO 14), or 24 (SEQ ID NO 16).

5 The HAP proteins of the present invention have limited homology to *Haemophilus influenzae* and *N. gonorrhoeae* serine-type IgA1 proteases. This homology, shown in Figure 7, is approximately 30-35% at the amino acid level, with several stretches showing 55-60% identity, including amino acids 457-549, 399-466, 572-622, and 233-261. However, the homology between the HAP protein and the IgA1 protease is considerably lower than the
10 similarity among the IgA1 proteases themselves.

In addition, the full length HAP protein has homology to Tsh, a hemagglutinin expressed by an avian *E. coli* strain (Provence and Curtiss 1994, Infect. Immun. 62:1369-1380). The homology is greatest in the N-terminal half of the proteins, and the overall homology is 30.5% homologous. The full length HAP protein also has homology with pertactin, a 69 kD outer
15 membrane protein expressed by *B. pertussis*, with the middle portion of the proteins showing 39% homology. HAP also has 34 - 52% homology with six regions of Hpma, a calcium-independent hemolysin expressed by *Proteus mirabilis* (Uphoff and Welch, 1990, J. Bacteriol. 172:1206-1216).

As used herein, a protein is a "HAP protein" if the overall homology of the protein sequence to one of the amino acid sequences shown in Figure 6, 11, 17, 19, 21, 23, or 25, is preferably greater than about 40 - 50%, more preferably greater than about 60% and most preferably greater than 80%. In some embodiments the homology will be as high as about 90 to 95 or 98%. This homology will be determined using standard techniques known in the art, such as the Best Fit sequence program described by Devereux *et al.*, *Nucl. Acid Res.* 12:387-395
20 (1984). The alignment may include the introduction of gaps in the sequences to be aligned. In addition, for sequences which contain either more or fewer amino acids than the proteins shown in Figure 6 or Figure 11, it is understood that the percentage of homology will be determined based on the number of homologous amino acids in relation to the total number of amino acids. Thus, for example, homology of sequences shorter than that shown in Figure 6
25 or 11, as discussed below, will be determined using the number of amino acids in the shorter sequence.

HAP proteins of the present invention may be shorter than the amino acid sequence shown in Figure 6 or 11. As shown in the Examples, the HAP protein may undergo post-translational processing similar to that seen for the serine-type IgA1 proteases expressed by *Haemophilus influenzae* and *N. gonorrhoeae*. These proteases are synthesized as preproteins with three
35 functional domains: the N-terminal signal peptide, the protease, and a C-terminal helper domain. Following movement of these proteins into the periplasmic space, the carboxy terminal β -domain of the proenzyme is inserted into the outer membrane, possibly forming a pore (Poulsen *et al.*, 1989, Infect. Immun. 57:3097-3105; Pohlner *et al.*, 1987, Nature (London). 325:458-462; Klauser *et al.*, 1992, EMBO J. 11:2327-2335; Klauser *et al.*, 1993, J. Mol. Biol. 234:579-593). Subsequently the amino end of the protein is exported through the outer membrane, and autoproteolytic cleavage occurs to result in secretion of the mature 100 to 106-kD protease. The 45 to 56-kD C-terminal β -domain remains associated with the outer
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membrane following the cleavage event. As shown in the Examples, the HAP nucleic acid is associated with expression of a 155 kD outer membrane protein. The secreted gene product is an approximately 110 kD protein, with the simultaneous appearance of a 45 kD outer membrane protein. The 45 kD protein corresponds to amino acids from about 1037 to about 1395 of Figure 6. Any one of these proteins is considered a HAP protein for the purposes of this invention.

Thus, in a preferred embodiment, included within the definition of HAP proteins are portions or fragments of the sequence shown in Figure 6 or 11. The fragments may be fragments of the entire sequence, the 110 kD sequence, or the 45 kD sequence. Generally, the HAP protein fragments may range in size from about 10 amino acids to about 1900 amino acids, with from about 50 to about 1000 amino acids being preferred, and from about 100 to about 500 amino acids also preferred. Particularly preferred fragments are sequences unique to HAP; these sequences have particular use in cloning HAP proteins from other organisms or to generate antibodies specific to HAP proteins. Unique sequences are easily identified by those skilled in the art after examination of the HAP protein sequence and comparison to other proteins; for example, by examination of the sequence alignment shown in Figure 7. For instance, as compared to the IgA proteases, unique sequences include, but are not limited to, amino acids 11-14, 16-22, 108-120, 155-164, 257-265, 281-288, 318-336, 345-353, 398-416, 684-693, 712-718, 753-761, 871-913, 935-953, 985-1008, 1023-1034, 1067-1076, 1440-1048, 1585-1592, 1631-1639, 1637-1648, 1735-1743, 1863-1871, 1882-1891, 1929-1941, and 1958-1966 (using the numbering of Figure 7). HAP protein fragments which are included within the definition of a HAP protein include N- or C-terminal truncations and deletions which still allow the protein to be biologically active; for example, which still exhibit proteolytic activity in the case of the 110 kD putative protease sequence. In addition, when the HAP protein is to be used to generate antibodies, for example as a vaccine, the HAP protein must share at least one epitope or determinant with either the full length protein, the 110 kD protein or the 45 kD protein, shown in Figure 6. In a preferred embodiment, the epitope is unique to the HAP protein; that is, antibodies generated to a unique epitope exhibit little or no cross-reactivity with other proteins. By "epitope" or "determinant" herein is meant a portion of a protein which will generate and/or bind an antibody. Thus, in most instances, antibodies made to a smaller HAP protein will be able to bind to the full length protein.

In a preferred embodiment the HAP protein contains sequences conserved among HAP proteins from different species or strains. As shown in Figure 11, alignment of the amino acid sequences of Hap TN106, HapP860295, and HapN187 revealed absolute identity through the first 47 amino acids, divergence over the next 350 amino acids, and then marked similarity over the final 1000-1050 amino acids. Accordingly, in a preferred embodiment the HAP protein of the invention includes amino acids 1-47 of the proteins shown in Figure 11. In another embodiment the HAP protein of the invention includes amino acids that are invariant among the sequences set forth in Figure 11 and contiguous for at least about 3, preferably at least about 5 and most preferably at least about 8 amino acids in length. Preferred peptides are included in the following Table 1.

TABLE 1

	SEQ. ID NO.	PEPTIDE SEQUENCE
5	18	MKKTVFRLNFLTACISLGIVSQAWAGHTYFGIDYQYYRDAENKGKF
	19	NPDQHRF
	20	GDSGSPMFIYD
10	21	INQGAGGLYFEGNG
	22	DRLSKIG
	23	YFGFRGGRDLNGHSLTF
15	24	RIQNTDEGAMIVNHN
	25	LLSSGGTNL
20	26	FSGRPTPHAYNHL
	27	RTFKAENFQIKGGSVVSRNVSSIEGNWTVSNNANA
	28	FGVVPNQNTICTRSDWTGLTTC
25	29	KVINSIP
	30	TQINGSINLTDNAT
30	31	GLAKLNGNVTL
	32	HSQFTLSNNATQ
	33	ATVDNANLNGNV
35	34	DSAQFSLKNSHFSHQIQG
	35	LENATWTMPSD
40	36	TLQNLTLNNST
	37	TLNSAYSA
45	38	RRSLETETTP TSAEHRFNTLTVNGKLSGQGTFQFTSSLFGYKSDKLSNDAE GDY
	39	LSVRNTGKEP
	40	QLTLVESKDN
50	41	FTLENDHVDAGALRYKLVKN
	42	GEFRLHNPIKEQEL
55	43	DLVRAEQAERTLEAKQVE
	44	LISRYSNSALSATSATVNSMLSVQDELDRLFVDQAQSAVWTNIAQDKRRYD SDAFRAYQQKTNL RQIGVQKAL
60	45	NGRIGAVFHSRSDNTFDEQVKNHATL
	46	MMSGFAQYQWGD LQFGVNVG
	47	GISASKMAEEQSRKIHRKAINYGVNASYQFRKGQLGIQPY
65	48	GVNRYFIERENYQSEEV

SEQ. ID NO.	PEPTIDE SEQUENCE
49	FNRNAGIRVDYTFTPTDNIS
50	KPYFFVNYVDVSNANVQTTVN
51	FGRYWQKEVGLKAEILHFQ
52	SAFISKSQGSQGLGKQQNVGVKLGYSRW

In some embodiments, the fragment of the HAP protein used to generate antibodies are small; thus, they may be used as haptens and coupled to protein carriers to generate antibodies, as is known in the art. In one embodiment the fragment of the HAP protein is a fragment of one of the peptides listed above. In this embodiment the fragment need only comprise a single epitope.

Accordingly in one embodiment the invention provides a composition comprising at least one of the peptides as shown in Table I. In addition the invention provides a polypeptide comprising at least one of the peptides as shown in Table I.

Preferably, the antibodies are generated to a portion of the HAP protein which remains attached to the *Haemophilus influenzae* organism. For example, the HAP protein can be used to vaccinate a patient to produce antibodies which upon exposure to the *Haemophilus influenzae* organism (e.g. during a subsequent infection) bind to the organism and allow an immune response. Thus, in one embodiment, the antibodies are generated to the roughly 45 kD fragment of the full length HAP protein. Preferably, the antibodies are generated to the portion of the 45 kD fragment which is exposed at the outer membrane.

In an alternative embodiment, the antibodies bind to the mature secreted 110 kD fragment. For example, as explained in detail below, the HAP proteins of the present invention may be administered therapeutically to generate neutralizing antibodies to the 110 kD putative protease, to decrease the undesirable effects and/or binding activity of the 100 kD fragment.

In the case of the nucleic acid, the overall homology of the nucleic acid sequence is commensurate with amino acid homology but takes into account the degeneracy in the genetic code and codon bias of different organisms. Accordingly, the nucleic acid sequence homology may be either lower or higher than that of the protein sequence. Thus the homology of the nucleic acid sequence as compared to the nucleic acid sequence of Figure 6, 16, 18, 20, 22 or 24, is preferably greater than 40%, more preferably greater than about 60% and most preferably greater than 80%. In some embodiments the homology will be as high as about 90 to 95 or 98%.

In one embodiment, the nucleic acid homology is determined through hybridization studies. Thus, for example, nucleic acids which hybridize under high stringency to all or part of the

nucleic acid sequence shown in Figure 6 are considered HAP protein genes. High stringency conditions include washes with 0.1XSSC at 65°C for 2 hours.

5 In one embodiment the nucleic acid of the invention are preferably greater than 40%, more preferably greater than about 60% and most preferably greater than 80% identical to the nucleic acids as set forth in Figure 6, 16, 18, 20, 22 or 24. In some embodiments the identity will be as high as about 90 to 95 or 98% up to 100%.

10 The HAP proteins and nucleic acids of the present invention are preferably recombinant. As used herein, "nucleic acid" may refer to either DNA or RNA, or molecules which contain both deoxy- and ribonucleotides. The nucleic acids include genomic DNA, cDNA and oligonucleotides including sense and anti-sense nucleic acids. Specifically included within the definition of nucleic acid are anti-sense nucleic acids. An anti-sense nucleic acid will hybridize to the corresponding non-coding strand of the nucleic acid sequence shown in Figure 6, but may contain ribonucleotides as well as deoxyribonucleotides. Generally, anti-sense nucleic acids function to prevent expression of mRNA, such that a HAP protein is not made, or made at reduced levels. The nucleic acid may be double stranded, single stranded, or contain portions of both double stranded or single stranded sequence. By the term "recombinant nucleic acid" herein is meant nucleic acid, originally formed in vitro by the manipulation of nucleic acid by endonucleases, in a form not normally found in nature. Thus an isolated HAP protein gene, in a linear form, or an expression vector formed in vitro by ligating DNA molecules that are not normally joined, are both considered recombinant for the purposes of this invention. It is understood that once a recombinant nucleic acid is made and reintroduced into a host cell or organism, it will replicate non-recombinantly, i.e. using the in vivo cellular machinery of the host cell rather than in vitro manipulations; however, such nucleic acids, once produced recombinantly, although subsequently replicated non-recombinantly, are still considered recombinant for the purposes of the invention.

30 Similarly, a "recombinant protein" is a protein made using recombinant techniques, i.e. through the expression of a recombinant nucleic acid as depicted above. A recombinant protein is distinguished from naturally occurring protein by at least one or more characteristics. For example, the protein may be isolated away from some or all of the proteins and compounds with which it is normally associated in its wild type host, or found in the absence of the host cells themselves. Thus, the protein may be partially or substantially purified. The definition includes the production of a HAP protein from one organism in a different organism or host cell. Alternatively, the protein may be made at a significantly higher concentration than is normally seen, through the use of an inducible promoter or high expression promoter, such that the protein is made at increased concentration levels. Alternatively, the protein may be in a form not normally found in nature, as in the addition of an epitope tag or amino acid substitutions, insertions and deletions.

40 Also included with the definition of HAP protein are HAP proteins from other organisms, including, but not limited to various *H. influenza* strains, which are cloned and expressed as outlined below.

In the case of anti-sense nucleic acids, an anti-sense nucleic acid is defined as one which will hybridize to all or part of the corresponding non-coding sequence of the sequence shown in

Figure 6. Generally, the hybridization conditions used for the determination of anti-sense hybridization will be high stringency conditions, such as 0.1XSSC at 65°C.

Once the HAP protein nucleic acid is identified, it can be cloned and, if necessary, its constituent parts recombined to form the entire HAP protein nucleic acid. Once isolated from its natural source, e.g., contained within a plasmid or other vector or excised therefrom as a linear nucleic acid segment, the recombinant HAP protein nucleic acid can be further used as a probe to identify and isolate other HAP protein nucleic acids. It can also be used as a "precursor" nucleic acid to make modified or variant HAP protein nucleic acids and proteins.

Using the nucleic acids of the present invention which encode HAP protein, a variety of expression vectors are made. The expression vectors may be either self-replicating extrachromosomal vectors or vectors which integrate into a host genome. Generally, these expression vectors include transcriptional and translational regulatory nucleic acid operably linked to the nucleic acid encoding the HAP protein. "Operably linked" in this context means that the transcriptional and translational regulatory DNA is positioned relative to the coding sequence of the HAP protein in such a manner that transcription is initiated. Generally, this will mean that the promoter and transcriptional initiation or start sequences are positioned 5' to the HAP protein coding region. The transcriptional and translational regulatory nucleic acid will generally be appropriate to the host cell used to express the HAP protein; for example, transcriptional and translational regulatory nucleic acid sequences from Bacillus will be used to express the HAP protein in Bacillus. Numerous types of appropriate expression vectors, and suitable regulatory sequences are known in the art for a variety of host cells.

In general, the transcriptional and translational regulatory sequences may include, but are not limited to, promoter sequences, leader or signal sequences, ribosomal binding sites, transcriptional start and stop sequences, translational start and stop sequences, and enhancer or activator sequences. In a preferred embodiment, the regulatory sequences include a promoter and transcriptional start and stop sequences.

Promoter sequences encode either constitutive or inducible promoters. The promoters may be either naturally occurring promoters or hybrid promoters. Hybrid promoters, which combine elements of more than one promoter, are also known in the art, and are useful in the present invention.

In addition, the expression vector may comprise additional elements. For example, the expression vector may have two replication systems, thus allowing it to be maintained in two organisms, for example in mammalian or insect cells for expression and in a procaryotic host for cloning and amplification. Furthermore, for integrating expression vectors, the expression vector contains at least one sequence homologous to the host cell genome, and preferably two homologous sequences which flank the expression construct. The integrating vector may be directed to a specific locus in the host cell by selecting the appropriate homologous sequence for inclusion in the vector. Constructs for integrating vectors are well known in the art.

In addition, in a preferred embodiment, the expression vector contains a selectable marker gene to allow the selection of transformed host cells. Selection genes are well known in the art and will vary with the host cell used.

5 The HAP proteins of the present invention are produced by culturing a host cell transformed with an expression vector containing nucleic acid encoding a HAP protein, under the appropriate conditions to induce or cause expression of the HAP protein. The conditions appropriate for HAP protein expression will vary with the choice of the expression vector and the host cell, and will be easily ascertained by one skilled in the art through routine experimentation. For example, the use of constitutive promoters in the expression vector will
10 require optimizing the growth and proliferation of the host cell, while the use of an inducible promoter requires the appropriate growth conditions for induction. In addition, in some embodiments, the timing of the harvest is important. For example, the baculoviral systems used in insect cell expression are lytic viruses, and thus harvest time selection can be crucial for product yield.

15 Appropriate host cells include yeast, bacteria, archebacteria, fungi, and insect and animal cells, including mammalian cells. Of particular interest are Drosophila melanogaster cells, Saccharomyces cerevisiae and other yeasts, E. coli, Bacillus subtilis, SF9 cells, C129 cells, 293 cells, Neurospora, BHK, CHO, COS, and HeLa cells, immortalized mammalian myeloid and lymphoid cell lines.

20 In a preferred embodiment, HAP proteins are expressed in bacterial systems. Bacterial expression systems are well known in the art.

A suitable bacterial promoter is any nucleic acid sequence capable of binding bacterial RNA polymerase and initiating the downstream (3') transcription of the coding sequence of HAP protein into mRNA. A bacterial promoter has a transcription initiation region which is usually
25 placed proximal to the 5' end of the coding sequence. This transcription initiation region typically includes an RNA polymerase binding site and a transcription initiation site. Sequences encoding metabolic pathway enzymes provide particularly useful promoter sequences. Examples include promoter sequences derived from sugar metabolizing enzymes, such as galactose, lactose and maltose, and sequences derived from biosynthetic
30 enzymes such as tryptophan. Promoters from bacteriophage may also be used and are known in the art. In addition, synthetic promoters and hybrid promoters are also useful; for example, the *tac* promoter is a hybrid of the *trp* and *lac* promoter sequences. Furthermore, a bacterial promoter can include naturally occurring promoters of non-bacterial origin that have the ability to bind bacterial RNA polymerase and initiate transcription.

35 In addition to a functioning promoter sequence, an efficient ribosome binding site is desirable. In *E. coli*, the ribosome binding site is called the Shine-Delgarno (SD) sequence and includes an initiation codon and a sequence 3-9 nucleotides in length located 3 - 11 nucleotides upstream of the initiation codon.

40 The expression vector may also include a signal peptide sequence that provides for secretion of the HAP protein in bacteria. The signal sequence typically encodes a signal peptide comprised of hydrophobic amino acids which direct the secretion of the protein from the cell,

as is well known in the art. The protein is either secreted into the growth media (gram-positive bacteria) or into the periplasmic space, located between the inner and outer membrane of the cell (gram-negative bacteria).

5 The bacterial expression vector may also include a selectable marker gene to allow for the selection of bacterial strains that have been transformed. Suitable selection genes include genes which render the bacteria resistant to drugs such as ampicillin, chloramphenicol, erythromycin, kanamycin, neomycin and tetracycline. Selectable markers also include biosynthetic genes, such as those in the histidine, tryptophan and leucine biosynthetic pathways.

10 These components are assembled into expression vectors. Expression vectors for bacteria are well known in the art, and include vectors for *Bacillus subtilis*, *E. coli*, *Streptococcus cremoris*, and *Streptococcus lividans*, among others.

The bacterial expression vectors are transformed into bacterial host cells using techniques well known in the art, such as calcium chloride treatment, electroporation, and others.

15 In one embodiment, HAP proteins are produced in insect cells. Expression vectors for the transformation of insect cells, and in particular, baculovirus-based expression vectors, are well known in the art. Briefly, baculovirus is a very large DNA virus which produces its coat protein at very high levels. Due to the size of the baculoviral genome, exogenous genes must be placed in the viral genome by recombination. Accordingly, the components of the expression system include: a transfer vector, usually a bacterial plasmid, which contains both a fragment of the baculovirus genome, and a convenient restriction site for insertion of the HAP protein; a wild type baculovirus with a sequence homologous to the baculovirus-specific fragment in the transfer vector (this allows for the homologous recombination of the heterologous gene into the baculovirus genome); and appropriate insect host cells and growth media.

25 Mammalian expression systems are also known in the art and are used in one embodiment. A mammalian promoter is any DNA sequence capable of binding mammalian RNA polymerase and initiating the downstream (3') transcription of a coding sequence for HAP protein into mRNA. A promoter will have a transcription initiating region, which is usually place proximal to the 5' end of the coding sequence, and a TATA box, using a located 25-30 base pairs upstream of the transcription initiation site. The TATA box is thought to direct RNA polymerase II to begin RNA synthesis at the correct site. A mammalian promoter will also contain an upstream promoter element, typically located within 100 to 200 base pairs upstream of the TATA box. An upstream promoter element determines the rate at which transcription is initiated and can act in either orientation. Of particular use as mammalian promoters are the promoters from mammalian viral genes, since the viral genes are often highly expressed and have a broad host range. Examples include the SV40 early promoter, mouse mammary tumor virus LTR promoter, adenovirus major late promoter, and herpes simplex virus promoter.

40 Typically, transcription termination and polyadenylation sequences recognized by mammalian cells are regulatory regions located 3' to the translation stop codon and thus, together with the promoter elements, flank the coding sequence. The 3' terminus of the mature mRNA is

formed by site-specific post-translational cleavage and polyadenylation. Examples of transcription terminator and polyadenylation signals include those derived from SV40.

5 The methods of introducing exogenous nucleic acid into mammalian hosts, as well as other hosts, is well known in the art, and will vary with the host cell used. Techniques include dextran-mediated transfection, calcium phosphate precipitation, polybrene mediated transfection, protoplast fusion, electroporation, encapsulation of the polynucleotide(s) in liposomes, and direct microinjection of the DNA into nuclei.

10 In a preferred embodiment, HAP protein is produced in yeast cells. Yeast expression systems are well known in the art, and include expression vectors for Saccharomyces cerevisiae, Candida albicans and C. maltosa, Hansenula polymorpha, Kluyveromyces fragilis and K. lactis, Pichia guilliermondii and P. pastoris, Schizosaccharomyces pombe, and Yarrowia lipolytica. Preferred promoter sequences for expression in yeast include the inducible GAL1,10 promoter, the promoters from alcohol dehydrogenase, enolase, glucokinase, glucose-6-phosphate isomerase, glyceraldehyde-3-phosphate-dehydrogenase, hexokinase, 15 phosphofructokinase, 3-phosphoglycerate mutase, pyruvate kinase, and the acid phosphatase gene. Yeast selectable markers include ADE2, HIS4, LEU2, TRP1, and ALG7, which confers resistance to tunicamycin; the G418 resistance gene, which confers resistance to G418; and the CUP1 gene, which allows yeast to grow in the presence of copper ions.

20 A recombinant HAP protein may be expressed intracellularly or secreted. The HAP protein may also be made as a fusion protein, using techniques well known in the art. Thus, for example, if the desired epitope is small, the HAP protein may be fused to a carrier protein to form an immunogen. Alternatively, the HAP protein may be made as a fusion protein to increase expression.

25 Also included within the definition of HAP proteins of the present invention are amino acid sequence variants. These variants fall into one or more of three classes: substitutional, insertional or deletional variants. These variants ordinarily are prepared by site specific mutagenesis of nucleotides in the DNA encoding the HAP protein, using cassette mutagenesis or other techniques well known in the art, to produce DNA encoding the variant, and thereafter expressing the DNA in recombinant cell culture as outlined above. However, 30 variant HAP protein fragments having up to about 100-150 residues may be prepared by in vitro synthesis using established techniques. Amino acid sequence variants are characterized by the predetermined nature of the variation, a feature that sets them apart from naturally occurring allelic or interspecies variation of the HAP protein amino acid sequence. The variants typically exhibit the same qualitative biological activity as the naturally occurring analogue, although variants can also be selected which have modified characteristics as will 35 be more fully outlined below.

40 While the site or region for introducing an amino acid sequence variation is predetermined, the mutation per se need not be predetermined. For example, in order to optimize the performance of a mutation at a given site, random mutagenesis may be conducted at the target codon or region and the expressed HAP protein variants screened for the optimal combination of desired activity. Techniques for making substitution mutations at predetermined sites in DNA having a known sequence are well known, for example, PCR

primer mutagenesis. Screening of the mutants is done using assays of HAP protein activities; for example, mutated HAP genes are placed in HAP deletion strains and tested for HAP activity, as disclosed herein. The creation of deletion strains, given a gene sequence, is known in the art. For example, nucleic acid encoding the variants may be expressed in a *Haemophilus influenzae* strain deficient in the HAP protein, and the adhesion and infectivity of the variant *Haemophilus influenzae* evaluated. Alternatively, the variant HAP protein may be expressed and its biological characteristics evaluated, for example its proteolytic activity.

Amino acid substitutions are typically of single residues; insertions usually will be on the order of from about 1 to 20 amino acids, although considerably larger insertions may be tolerated. Deletions range from about 1 to 30 residues, although in some cases deletions may be much larger, as for example when one of the domains of the HAP protein is deleted.

Substitutions, deletions, insertions or any combination thereof may be used to arrive at a final derivative. Generally these changes are done on a few amino acids to minimize the alteration of the molecule. However, larger changes may be tolerated in certain circumstances.

When small alterations in the characteristics of the HAP protein are desired, substitutions are generally made in accordance with the following chart:

Chart I

	<u>Original Residue</u>	<u>Exemplary Substitutions</u>
20	Ala	Ser
	Arg	Lys
	Asn	Gln, His
	Asp	Glu
	Cys	Ser
25	Gln	Asn
	Glu	Asp
	Gly	Pro
	His	Asn, Gln
	Ile	Leu, Val
30	Leu	Ile, Val
	Lys	Arg, Gln, Glu
	Met	Leu, Ile
	Phe	Met, Leu, Tyr
	Ser	Thr
35	Thr	Ser
	Trp	Tyr
	Tyr	Trp, Phe
	Val	Ile, Leu

Substantial changes in function or immunological identity are made by selecting substitutions that are less conservative than those shown in Chart I. For example, substitutions may be made which more significantly affect: the structure of the polypeptide backbone in the area of the alteration, for example the alpha-helical or beta-sheet structure; the charge or hydrophobicity of the molecule at the target site; or the bulk of the side chain. The substitutions which in general are expected to produce the greatest changes in the polypeptide's properties are those in which (a) a hydrophilic residue, e.g. seryl or threonyl, is substituted for (or by) a hydrophobic residue, e.g. leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue

having an electropositive side chain, e.g. lysyl, arginyl, or histidyl, is substituted for (or by) an electronegative residue, e.g. glutamyl or aspartyl; or (d) a residue having a bulky side chain, e.g. phenylalanine, is substituted for (or by) one not having a side chain, e.g. glycine.

5 The variants typically exhibit the same qualitative biological activity and will elicit the same immune response as the naturally-occurring analogue, although variants also are selected to modify the characteristics of the polypeptide as needed. Alternatively, the variant may be designed such that the biological activity of the HAP protein is altered. For example, the proteolytic activity of the larger 110 kD domain of the HAP protein may be altered, through the substitution of the amino acids of the active site. The putative catalytic domain of this protein was considered to be GDSGSPMF (SEQ ID NO: 53). The residues of the active site may be individually or simultaneously altered to decrease or eliminate proteolytic activity. 10 This may be done to decrease the toxicity or side effects of the vaccine. Similarly, the cleavage site between the 45 kD domain and the 100 kD domain may be altered, for example to eliminate proteolytic processing to form the two domains. Indeed, the catalytic triad has been defined as His98, Asp 140 and Ser 243. Each of these amino acids has been mutated; the mutations eliminated proteolytic activity. In addition, four sites have been identified at which autoproteolytic cleavage occurs (Hendrixson et al., 1997; Hendrixson and St. Geme, 1998; Fink et al., 2001).

20 In a preferred embodiment, the HAP protein is purified or isolated after expression. HAP proteins may be isolated or purified in a variety of ways known to those skilled in the art depending on what other components are present in the sample. Standard purification methods include electrophoretic, molecular, immunological and chromatographic techniques, including ion exchange, hydrophobic, affinity, and reverse-phase HPLC chromatography, and chromatofocusing. For example, the HAP protein may be purified using a standard anti-HAP antibody column. Ultrafiltration and diafiltration techniques, in conjunction with protein concentration, are also useful. For general guidance in suitable purification techniques, see 25 Scopes, R., Protein Purification, Springer-Verlag, NY (1982). The degree of purification necessary will vary depending on the use of the HAP protein. In some instances no purification will be necessary.

30 Once expressed and purified if necessary, the HAP proteins are useful in a number of applications.

For example, the HAP proteins can be coupled, using standard technology, to affinity chromatography columns. These columns may then be used to purify antibodies from samples obtained from animals or patients exposed to the *Haemophilus influenzae* 35 organism. The purified antibodies may then be used as outlined below.

40 Additionally, the HAP proteins are useful to make antibodies to HAP proteins. These antibodies find use in a number of applications. In a preferred embodiment, the antibodies are used to diagnose the presence of an *Haemophilus influenzae* infection in a sample or patient. This will be done using techniques well known in the art; for example, samples such as blood or tissue samples may be obtained from a patient and tested for reactivity with the antibodies, for example using standard techniques such as ELISA. In a preferred embodiment, monoclonal antibodies are generated to the HAP protein, using techniques well

known in the art. As outlined above, the antibodies may be generated to the full length HAP protein, or a portion of the HAP protein.

Antibodies generated to HAP proteins may also be used in passive immunization treatments, as is known in the art.

5 Antibodies generated to unique sequences of HAP proteins may also be used to screen expression libraries from other organisms to find, and subsequently clone, HAP nucleic acids from other organisms.

10 In one embodiment, the antibodies may be directly or indirectly labelled. By "labelled" herein is meant a compound that has at least one element, isotope or chemical compound attached to enable the detection of the compound. In general, labels fall into three classes: a) isotopic labels, which may be radioactive or heavy isotopes; b) immune labels, which may be antibodies or antigens; and c) colored or fluorescent dyes. The labels may be incorporated into the compound at any position. Thus, for example, the HAP protein antibody may be labelled for detection, or a secondary antibody to the HAP protein antibody may be created and labelled.

15 In one embodiment, the antibodies generated to the HAP proteins of the present invention are used to purify or separate HAP proteins or the *Haemophilus influenzae* organism from a sample. Thus for example, antibodies generated to HAP proteins which will bind to the *Haemophilus influenzae* organism may be coupled, using standard technology, to affinity chromatography columns. These columns can be used to pull out the *Haemophilus* organism from environmental or tissue samples. Alternatively, antibodies generated to the soluble 110 kD portion of the full-length portion of the protein shown in Figure 7 may be used to purify the 110 kD protein from samples.

20 In a preferred embodiment, the HAP proteins of the present invention are used as vaccines for the prophylactic or therapeutic treatment of a *Haemophilus influenzae* infection in a patient. By "vaccine" herein is meant an antigen or compound which elicits an immune response in an animal or patient. The vaccine may be administered prophylactically, for example to a patient never previously exposed to the antigen, such that subsequent infection by the *Haemophilus influenzae* organism is prevented. Alternatively, the vaccine may be administered therapeutically to a patient previously exposed or infected by the *Haemophilus influenzae* organism. While infection cannot be prevented, in this case an immune response is generated which allows the patient's immune system to more effectively combat the infection. Thus, for example, there may be a decrease or lessening of the symptoms associated with infection.

25 In a preferred embodiment the HAP proteins of the invention protect against infection by *H. influenza*. That is, administration of at least one of the HAP proteins of the invention to a patient results in protection against *H. influenza* infection. In another embodiment administration of at least one HAP protein of the invention results in reduced colonization by *H. influenza*. In a particularly preferred embodiment administration of at least one of the HAP proteins of the invention results in protection against infection or colonization by a heterologous strain of *Haemophilus influenzae*. By "heterologous" is meant a strain that is

not the same strain from which the HAP protein is obtained. Accordingly, the present invention provides a method of vaccinating against infection by a heterologous strain of *H. influenza*.

5 A "patient" for the purposes of the present invention includes both humans and other animals and organisms. Thus the methods are applicable to both human therapy and veterinary applications.

10 The administration of the HAP protein as a vaccine is done in a variety of ways, including but not limited to intramuscular or subcutaneous injection, intranasal delivery, oral delivery, intravenous delivery and intradermal delivery as is known in the art. Generally, the HAP proteins can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby therapeutically effective amounts of the HAP protein are combined in admixture with a pharmaceutically acceptable carrier vehicle. Suitable vehicles and their formulation are well known in the art. Such compositions will contain an effective amount of the HAP protein together with a suitable amount of vehicle in order to prepare
15 pharmaceutically acceptable compositions for effective administration to the host. The composition may include salts, buffers, carrier proteins such as serum albumin, targeting molecules to localize the HAP protein at the appropriate site or tissue within the organism, and other molecules. The composition may include adjuvants as well. In a preferred embodiment the HAP protein is administered combined with an adjuvant as is known in the art, such as aluminum hydroxide. In a preferred embodiment the adjuvant is a modified cholera toxin adjuvant.
20

Cholera toxin (CT) is well known as a potent mucosal adjuvant but is highly toxic to humans (Snider, D.P., 1995, Crit. Rev. Immunol 15:317-48). CT-E29H is a mutant form of CT that contains a histidine in place of a glutamic acid at residue 29 in the enzymatic A subunit. This mutant lacks enzymatic activity and has <1% of the cellular toxicity of native cholera toxin but remains fully active as an adjuvant, suggesting considerable utility in humans (Tebbey *et al.*, 2000, Vaccine 18:2723-34). Accordingly, in a preferred embodiment the invention provides a composition comprising a HAP protein of the invention and cholera toxin CT-E29H. In addition the invention provides a method of improving immunization by
25 administering an immunogenic protein of the invention and an adjuvant. In a preferred embodiment the adjuvant is CT-E29H.
30

In one embodiment, the vaccine is administered as a single dose; that is, one dose is adequate to induce a sufficient immune response to prophylactically or therapeutically treat a *Haemophilus influenzae* infection. In alternate embodiments, the vaccine is administered
35 as several doses over a period of time, as a primary vaccination and "booster" vaccinations.

By "therapeutically effective amounts" herein is meant an amount of the HAP protein which is sufficient to induce an immune response. This amount may be different depending on whether prophylactic or therapeutic treatment is desired. Generally, this ranges from about 0.001 mg to about 1 gm, with a preferred range of about 0.05 mg to about 0.5 g, and the preferred dose being 0.1mg to about 0.1 g These amounts may be adjusted if adjuvants are used.
40

The following examples serve to more fully describe the manner of using the above-described invention, as well as to set forth the best modes contemplated for carrying out various aspects of the invention. It is understood that these examples in no way serve to limit the true scope of this invention, but rather are presented for illustrative purposes.

EXAMPLES

Example 1 Cloning of the HAP protein

Bacterial Strains, plasmids, and phage. *H. influenzae* strain N187 is a clinical isolate that was originally cultivated from the middle ear fluid of a child with acute otitis media. This strain was classified as nontypable based on the absence of agglutination with typing antisera for *H. influenzae* types a-f (Burroughs Wellcome) and the failure to hybridize with pU038, a plasmid that contains the entire *cap b* locus (Kroll and Moxon, 1988, J. Bacteriol. 170:859-864).

H. influenzae strain DB117 is a *rec-1* mutant of Rd, a capsule-deficient serotype d strain that has been in the laboratory for over 40 years (Alexander and Leidy, 1951, J. Exp. Med. 83:345-359); DB117 was obtained from G. Barcak (University of Maryland, Baltimore, MD) (Sellow *et al.*, 1968). DB117 is deficient for *in vitro* adherence and invasion, as assayed below.

H. influenzae strain 12 is the nontypable strain from which the genes encoding the HMW1 and HMW2 proteins were cloned (Barenkamp and Leininger, 1992, Infect. Immun. 60:1302-1313); HMW1 and HMW2 are the prototypic members of a family of nontypable *Haemophilus* antigenically-related high-molecular-weight adhesive proteins (St. Geme *et al.*, 1993).

E. coli HB101, which is nonadherent and noninvasive, has been previously described (Sambrook *et al.*, 1989, Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). *E. coli* DH5 α was obtained from Bethesda Research Laboratories. *E. coli* MC1061 was obtained from H. Kimsey (Tufts University, Boston, MA). *E. coli* XL-1 Blue and the plasmid pBluescript KS- were obtained from Stratagene. Plasmid pT7-7 and phage mGP1-2 were provided by S. Tabor (Harvard Medical School, Boston, MA) (Tabor and Richardson, 1985, Proc. Natl. Acad. Sci. USA. 82:1074-1078). The *E. coli*-*Haemophilus* shuttle vector pGJB103 (Tomb *et al.*, 1989, Rd. J. Bacteriol. 171:3796-3802) and phage λ 1105 (Way *et al.*, 1984, Gene. 32:3 69-379) were provided by G. Barcak (University of Maryland, Baltimore, MD). Plasmid pVD116 harbors the IgA1 protease gene from *H. influenzae* strain Rd (Kooimey and Falkow, 1984, Infect. Immun. 43:101-107) and was obtained from M. Kooimey (University of Michigan, Ann Arbor, MI).

Growth conditions. *H. influenzae* strains were grown as described (Anderson *et al.*, 1972, J. Clin. Invest. 51:31-38). They were stored at -80°C in brain heart infusion broth with 25% glycerol. *E. coli* strains were grown on LB agar or in LB broth. They were stored at -80°C in LB broth with 50% glycerol.

For *H. influenzae*, tetracycline was used in a concentration of 5 µg/ml and kanamycin was used in a concentration of 25 µg/ml. For *E. coli*, antibiotics were used in the following concentrations: tetracycline, 12.5 µg/ml; kanamycin, 50 µg/ml; ampicillin, 100 µg/ml.

Recombinant DNA methods. DNA ligations, restriction endonuclease digestions, and gel electrophoresis were performed according to standard techniques (Sambrook *et al.*, 1989, *supra*). Plasmids were introduced into *E. coli* strains by either chemical transformation or electroporation, as described (Sambrook *et al.*, 1989, *supra*; Dower *et al.*, 1988, *Nucleic Acids Res.* 16:6127-6145). In *H. influenzae* transformation was performed using the MIV method of Herriott *et al.* (1970, *J. Bacteriol.* 101:517-524), and electroporation was carried out using the protocol developed for *E. coli* (Dower *et al.*, 1988, *supra*).

Construction of genomic library from *H. influenzae* strain N187. High-molecular-weight chromosomal DNA was prepared from 3 ml of an overnight broth culture of *H. influenzae* N187 as previously described (Mekalanos, 1983, *Cell.* 35:253-263). Following partial digestion with *Sau3A*I, 8 to 12 kb fragments were eluted into DEAE paper (Schleicher & Schuell, Keene, H.H.) and then ligated to *Bgl*II-digested calf intestine phosphatase-treated pGJB103. The ligation mixture was electroporated into *H. influenzae* DB117, and transformants were selected on media containing tetracycline.

Transposon mutagenesis. Mutagenesis of plasmid DNA was performed using the mini-Tn10 *kan* element described by Way *et al.* (1984, *supra*). Initially, the appropriate plasmid was introduced into *E. coli* MC1061. The resulting strain was infected with λ1105, which carries the mini-Tn10 *kan* transposon. Transductants were grown overnight in the presence of kanamycin and an antibiotic to select for the plasmid, and plasmid DNA was isolated using the alkaline lysis method. In order to recover plasmids containing a transposon insertion, plasmid DNA was electroporated into *E. coli* DH5α, plating on media containing kanamycin and the appropriate second antibiotic.

In order to establish more precisely the region of pN187 involved in promoting interaction with host cells, initially this plasmid was subjected to restriction endonuclease analysis. Subsequently, several subclones were constructed in the vector pGJB103 and were reintroduced into *H. influenzae* strain DB117. The resulting strains were then examined for adherence and invasion. As summarized in Figure 4, subclones containing either a 3.9-kb *Pst*I-*Bgl*II fragment (pJS105) or the adjoining 4.2-kb *Bgl*II fragment (pJS102) failed to confer the capacity to associate with Chang cells. In contrast, a subclone containing an insert that included portions of both of these fragments (pJS106) did promote interaction with epithelial monolayers. Transposon mutagenesis performed on pN187 confirmed that the flanking portions of the insert in this plasmid were not required for the adherent/invasive phenotype. On the other hand, a transposon insertion located adjacent to the *Bgl*II site in pJS106 eliminated adherence and invasion. An insertion between the second *Eco*RI and *Pst*I sites in this plasmid had a similar effect (Figure 4).

Examination of plasmid-encoded proteins. In order to examine plasmid encoded proteins, relevant DNA was ligated into the bacteriophage T7 expression vector pT7-7, and the resulting construct was transformed into *E. coli* XL-1 Blue. Plasmid pT7-7 contains the T7 phage φ10 promoter and ribosomal binding site upstream of a multiple cloning site

(Tabor and Richardson, 1985, *supra*). The T7 promoter was induced by infection with the recombinant M13 phage mGP1-2 and addition of isopropyl- β -D-thiogalactopyranoside (final concentration, 1 mM). Phage mGP1-2 contains the gene encoding T7 RNA polymerase, which activates the ϕ 10 promoter in pT7-7 (Tabor and Richardson, 1985, *supra*).

5 Like DB117(pN187), strain DB117 carrying pJS106 expressed new outer membrane proteins 160-kD and 45-kD in size (Figure 3, lane 3). In order to examine whether the 6.5-kb insert in pJS106 actually encodes these proteins, this fragment of DNA was ligated into the bacteriophage T7 expression vector pT7-7. The resulting plasmid containing the insert in the same orientation as in pN187 was designated pJS104, and the plasmid with the insert in
10 the opposite orientation was designated pJS103. Both pJS104, and pJS103 were introduced into *E. coli* XL-1 Blue, producing XL-1 Blue(pJS104) and XL-1 Blue(pJS103), respectively. As a negative control, pT7-7 was also transformed into XL-1 Blue. The T7 promoter was induced in these three strains by infection with the recombinant M13 phage mGP1-2 and addition of isopropyl- β -D-thiogalactopyranoside (final concentration, 1 mM),
15 and induced proteins were detected using [35 S] methionine. As shown in Figure 5, induction of XL-1 Blue(pJS104) resulted in expression of a 160-kD protein and several smaller proteins which presumably represent degradation products. In contrast, when XL-1 Blue(pJS103) and XL-1 Blue(pT7-7) were induced, there was no expression of these proteins. There was no 45-kD protein induced in any of the three strains. This experiment
20 suggested that the 6.5-kb insert present in pJS106 contains the structural gene for the 160-kD outer membrane protein identified in DB117(pJS106). On the other hand, this analysis failed to establish the origin of the 45-kD membrane protein expressed by DB117(pJS106).

Adherence and invasion assays. Adherence and invasion assays were performed with Chang epithelial cells [Wong-Kilbourne derivative, clone 1-5c-4 (human conjunctiva)], which
25 were seeded into wells of 24-well tissue culture plates as previously described (St. Geme and Falkow, 1990). Adherence was measured after incubating bacteria with epithelial monolayers for 30 minutes as described (St. Geme *et al.*, 1993). Invasion assays were carried out according to our original protocol and involved incubating bacteria with epithelial cells for four hours followed by treatment with gentamicin for two hours (100 μ g/ml) (St.
30 Geme and Falkow, 1990).

Nucleotide sequence determination and analysis. Nucleotide sequence was determined using a Sequenase kit and double stranded plasmid template. DNA fragments were subcloned into pBluescript KS⁻ and sequenced along both strands by primer walking. DNA
35 sequence analysis was performed using the Genetics Computer Group (GCG) software package from the University of Wisconsin (Devereux *et al.*, 1984). Sequence similarity searches were carried out using the BLAST program of the National Center for Biotechnology Information (Altschul *et al.*, 1990, *J. Mol. Biol.* 215:403-410). The DNA sequence described here will be deposited in the EMBL/GenBank/DDBJ Nucleotide Sequence Data Libraries.

40 Based on the our subcloning results, we reasoned that the central *Bgl*II site in pN187 was positioned within an open reading frame. Examination of a series of mini-Tn10 *kan* mutants supported this conclusion (Figure 4). Consequently, we sequenced DHA on either side of this *Bgl*II site and identified a 4182 bp gene, which we have designated *hap* for *Haemophilus*

adherence and penetration (Figure 6). This gene encodes a 1394 amino acid polypeptide, which we have called Hap, with a calculated molecular mass of 155.4-kD, in good agreement with the molecular mass of the larger of the two novel outer membrane proteins expressed by DB117(pN187) and the protein expressed after induction of XL-1 Blue/pJS104. The *hap* gene has a G+C content of 39.1%, similar to the published estimate of 38.7% for the whole genome (Kilian, 1976, J. Gen. Microbiol. 93:9-62). Putative -10 and -35 promoter sequences are present upstream of the initiation codon. A consensus ribosomal binding site is lacking. A sequence similar to a *rho*-independent transcription terminator is present beginning 39 nucleotides beyond the stop codon and contains interrupted inverted repeats with the potential for forming a hairpin structure containing a loop of three bases and a stem of eight bases. Similar to the situation with typical *E. coli* terminators, this structure is followed by a stretch rich in T residues. Analysis of the predicted amino acid sequence suggested the presence of a 25 amino acid signal peptide at the amino terminus. This region has characteristics typical of procaryotic signal peptides, with three positive H-terminal charges, a central hydrophobic region, and alanine residues at positions 23 and 25 (-3 and -1 relative to the putative cleavage site) (von Heijne, 1984, J. Mol. Biol. 173:243-251).

Comparison of the deduced amino acid sequence of Hap with other proteins. A protein sequence similarity search was performed with the predicted amino acid sequence using the BLAST network service of the National Center for Biotechnology Information (Altschul *et al.*, 1990, *supra*). This search revealed homology with the IgA1 proteases of *H. influenzae* and *Neisseria gonorrhoeae*. Alignment of the derived amino acid sequences for the *hap* gene product and the IgA1 proteases from four different *H. influenzae* strains revealed homology across the extent of the proteins (Figure 7), with several stretches showing 55-60% identity and 70-80% similarity. Similar levels of homology were noted between the *hap* product and the IgA1 protease from *N. gonorrhoeae* strain MS11. This homology includes the region identified as the catalytic site of the IgA1 proteases, which is comprised of the sequence GDSGSPLF (SEQ ID NO: 54), where 2 is the active site serine characteristic of serine proteases (Brenner, 1988, Nature (London). 334:528-530; Poulsen *et al.*, 1992, J. Bacteriol. 174:2913-2921). In the case of Hap, the corresponding sequence is GDSGSPMF (SEQ ID NO: 53). The *hap* product also contains two cysteines corresponding to the cysteines proposed to be important in forming the catalytic domain of the IgA proteases (Pohlner *et al.*, 1987, *supra*). Overall there is 30-35% identity and 51-55% similarity between the *hap* gene product and the *H. influenzae* and *N. gonorrhoeae* IgA proteases.

The deduced amino acid sequence encoded by *hap* was also found to contain significant homology to Tsh, a hemagglutinin expressed by an avian *E. coli* strain (Provence and Curtiss, 1994, *supra*). This homology extends throughout both proteins but is greatest in the H-terminal half of each. Overall the two proteins are 30.5% identical and 51.6% similar. Tsh is also synthesized as a preprotein and is secreted as a smaller form; like the IgA1 proteases and perhaps Hap, a carboxy terminal peptide remains associated with the outer membrane (D. Provence, personal communication). While this protein is presumed to have proteolytic activity, its substrate has not yet been determined. Interestingly, Tsh was first identified on the basis of its capacity to promote agglutination of erythrocytes. Thus Hap and

Tsh are possibly the first members of a novel class of adhesive proteins that are processed analogously to the IgA1 proteases.

Homology was also noted with pertactin, a 69-kD outer membrane protein expressed by *B. pertussis* (Charles *et al.*, 1989, Proc. Natl. Acad. Sci. USA. 86:3554-3558). The middle portions of these two molecules are 39% identical and nearly 60% similar. This protein contains the amino acid triplet arginine-glycine-aspartic acid (RGD) and has been shown to promote attachment to cultured mammalian cells via this sequence (Leininger *et al.*, 1991, Proc. Natl. Acad. Sci. USA. 88:345-349). Although *Bordetella* species are not generally considered intracellular parasites, work by Ewanowich and coworkers indicates that these respiratory pathogens are capable of *in vitro* entry into human epithelial cells (Ewanowich *et al.*, 1989, Infect. Immun. 57:2698-2704; Ewanowich *et al.*, 1989, Infect. Immun. 57:1240-1247). Recently Leininger *et al.* reported that preincubation of epithelial monolayers with an RGD-containing peptide derived from the pertactin sequence specifically inhibited *B. pertussis* entry (Leininger *et al.*, 1992, Infect. Immun. 60:2380-2385). In addition, these investigators found that coating of *Staphylococcus aureus* with purified pertactin resulted in more efficient *S. aureus* entry; the RGD-containing peptide from pertactin inhibited this pertactin-enhanced entry by 75%. Although the *hap* product lacks an RGD motif, it is possible that Hap and pertactin serve similar biologic functions for *H. influenzae* and *Bordetella* species, respectively.

Additional analysis revealed significant homology (34 to 52% identity, 42 to 70% similarity) with six regions of HpmA, a calcium-independent hemolysin expressed by *Proteus mirabilis* (Uphoff and Welch, 1990, *supra*).

The *hap* locus is distinct from the *H. influenzae* IgA1 protease gene. Given the degree of similarity between the *hap* gene product and *H. influenzae* IgA1 protease, we wondered whether we had isolated the IgA1 protease gene of strain N187. To examine this possibility, we performed IgA1 protease activity assays. Among *H. influenzae* strains, two enzymatically distinct types of IgA1 protease have been found (Mulks *et al.*, 1982, J. Infect. Dis. 146:266-274). Type 1 enzymes cleave the Pro-Ser peptide bond between residues 231 and 232 in the hinge region of human IgA1 heavy chain and generate fragments of roughly 28-kD and 31-kD; type 2 enzymes cleave the Pro-Thr bond between residues 235 and 236 in the hinge region and generate 26.5-kD and 32.5-kD fragments. Previous studies of the parent strain from which DB117 was derived have demonstrated that this strain produces a type 1 IgA1 protease (Koomey and Falkow, 1984, *supra*). As shown in Figure 8, comparison of the proteolytic activities of strain DB117 and strain N187 suggested that N187 produces a type 2 IgA1 protease. We reasoned that DB117(pN187) might generate a total of four fragments from IgA1 protease, consistent with two distinct cleavage specificities. Examination of DB117(pN187) revealed instead that this transformant produces the same two fragments of the IgA1 heavy chain as does DB117, arguing that this strain produces only a type 1 enzyme.

In an effort to obtain additional evidence against the possibility that plasmid pN187 contains the N187 IgA1 protease gene, we performed a series of Southern blots. As shown in Figure 9, when genomic DNA from strain N187 was digested with *EcoRI*, *BglII*, or *BamHI* and then probed with the *hap* gene, one set of hybridizing fragments was detected. Probing of the

same DNA with the *iga* gene from *H. influenzae* strain Rd resulted in a different set of hybridizing bands. Moreover, the *iga* gene failed to hybridize with a purified 4.8-kb fragment that contained the intact *hap* gene.

The recombinant plasmid associated with adherence and invasion encodes a

secreted protein. The striking homology between the *hap* gene product and the *Haemophilus* and *Neisseria* IgA1 proteases suggested the possibility that these proteins might be processed in a similar manner. The IgA1 proteases are synthesized as preproteins with three functional domains: the N-terminal signal peptide, the protease, and a C-terminal helper domain, which is postulated to form a pore in the outer membrane for secretion of the protease (Poulsen *et al.*, 1989, *supra*; Pohlner *et al.*, 1987, *supra*). The C-terminal peptide remains associated with the outer membrane following an autoproteolytic cleavage event that results in release of the mature enzyme. Consistent with the possibility that the *hap* gene product follows a similar fate, we found that DB117(pN187) produced a secreted protein approximately 110-kD in size that was absent from DB117(pGJB103) (Figure 10). This protein was also produced by DB117(pJS106), but not by DB117(pJ5102) or DB117(pJS105). Furthermore, the two mutants with transposon insertions within the *hap* coding region were deficient in this protein. In order to determine the relationship between *hap* and the secreted protein, this protein was transferred to a PVDF membrane and N-terminal amino acid sequencing was performed. Excessive background on the first cycle precluded identification of the first amino acid residue of the free amino terminus. The sequence of the subsequent seven residues was found to be HTYFGID (SEQ ID NO: 55), which corresponds to amino acids 27 through 33 of the *hap* product.

The introduction of *hap* into laboratory strains of *E. coli* strains was unable to endow these organisms with the capacity for adherence or invasion. In considering these results, it is noteworthy that the *E. coli* transformants failed to express either the 160-kD or the 45-kD outer membrane protein. Accordingly, they also failed to express the 110-kD secreted protein. The explanation for this lack of expression is unclear. One possibility is that the *H. influenzae* promoter or ribosomal binding site was poorly recognized in *E. coli*. Indeed the putative -35 sequence upstream of the *hap* initiation codon is fairly divergent from the $\sigma 70$ consensus sequence, and the ribosomal binding site is unrecognizable. Alternatively, an accessory gene may be required for proper export of the Hap protein, although the striking homology with the IgA proteases, which are normally expressed and secreted in *E. coli*, argues against this hypothesis.

In considering the possibility that the *hap* gene product promotes adherence and invasion by directly binding to a host cell surface structure, it seems curious that the mature protein is secreted from the organism. However, there are examples of other adherence factors that are also secreted. Filamentous hemagglutinin is a 220-kD protein expressed by *B. pertussis* that mediates *in vitro* adherence and facilitates natural colonization (Relman *et al.*, 1989, *Proc. Natl. Acad. Sci. U.S.A.* 86:2637-2641; Kimura *et al.*, 1990, *Infect. Immun.* 58:7-16). This protein remains surface-associated to some extent but is also released from the cell. The process of Filamentous hemagglutinin secretion involves an accessory protein designated FhaC, which appears to be localized to the outer membrane (Willems *et al.*, 1994, *Molec. Microbiol.* 11:337-347). Similarly, the Ipa proteins implicated in *Shigella* invasion are also secreted. Secretion of these proteins requires the products of multiple

genes within the *mxl* and *spa* loci (Allaoui *et al.*, 1993, *Molec. Microbiol.* 7:59-68; Andrews *et al.*, 1991, *Infect. Immun.* 59:1997-2005; Venkatsan *et al.*, 1992, *J. Bacteriol.* 174:1990-2001).

It is conceivable that secretion is simply a consequence of the mechanism for export of the *hap* gene product to the surface of the organism. However, it is noteworthy that the secreted protein contains a serine-type protease catalytic domain and shows homology with the *P. mirabilis* hemolysin. These findings suggest that the mature Hap protein may possess proteolytic activity and raise the possibility that Hap promotes interaction with the host cell at a distance by modifying the host cell surface. Alternatively, Hap may modify the bacterial surface in order to facilitate interaction with a host cell receptor. It is possible that *hap* encodes a molecule with dual functions, serving as both adhesin and protease.

Analysis of outer membrane and secreted proteins. Outer membrane proteins were isolated on the basis of sarcosyl insolubility according to the method of Carlone *et al.* (1986, *J. Clin. Microbiol.* 24:330-332). Secreted proteins were isolated by centrifuging bacterial cultures at 16,000 g for 10 minutes, recovering the supernatant, and precipitating with trichloroacetic acid in a final concentration of 10%. SDS-polyacrylamide gel electrophoresis was performed as previously described (Laemmli, 1970, *Nature (London)*. 227:680-685).

To identify proteins that might be involved in the interaction with the host cell surface, outer membrane protein profiles for DB117(pN187) and DB117(pGJB103) were compared. As shown in Figure 3, DB117(pN187) expressed two new outer membrane proteins: a high-molecular-weight protein approximately 160-kD in size and a 45-kD protein. *E. coli* HB101 harboring pN187 failed to express these proteins, suggesting an explanation for the observation that HB101(pN187) is incapable of adherence or invasion.

Previous studies have demonstrated that a family of antigenically-related high-molecular-weight proteins with similarity to filamentous hemagglutinin of *Bordetella pertussis* mediate attachment by nontypable *H. influenzae* to cultured epithelial cells (St. Geme *et al.*, 1993). To explore the possibility that the gene encoding the strain H187 member of this family was cloned, whole cell lysates of N187, DB117(pN187), and DB117(pGJB103) were examined by Western immunoblot. Our control strain for this experiment was *H. influenzae* strain 12. Using a polyclonal antiserum directed against HMW1 and HMW2, the prototypic proteins in this family, we identified a 140-kD protein in strain H187 (not shown). In contrast, this antiserum failed to react with either DB117(pN187) or DB117(pGJB103) (not shown), indicating that pN187 has no relationship to HMW protein expression.

Determination of amino terminal sequence. Secreted proteins were precipitated with trichloroacetic acid, separated on a 10% SDS-polyacrylamide gel, and electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Matsudaira, 1987, *J. Biol. Chem.* 262:10035-10038). Following staining with Coomassie Brilliant Blue R-250, the 110-kD protein was cut from the PVDF membrane and submitted to the Protein Chemistry Laboratory at Washington University School of Medicine for amino terminal sequence determination. Sequence analysis was performed by automated Edman degradation using an Applied Biosystems Model 470A protein sequencer.

Examination of IgA1 protease activity. In order to assess IgA1 protease activity, bacteria were inoculated into broth and grown aerobically overnight. Samples were then centrifuged in a microfuge for two minutes, and supernatants were collected. A 10 µl volume of supernatant was mixed with 16 µl of 0.5 µg/ml human IgA1 (Calbiochem), and
5 chloramphenicol was added to a final concentration of 2 µg/ml. After overnight incubation at 37°C, reaction mixtures were electrophoresed on a 10% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with goat anti-human IgA1 heavy chain conjugated to alkaline phosphatase (Kirkegaard & Perry). The membrane was developed by immersion in phosphatase substrate solution (5-bromo-4-chloro-3-
10 indolylphosphate toluidinium-nitro blue tetrazolium substrate system; Kirkegaard & Perry).

Immunoblot analysis. Immunoblot analysis of bacterial whole cell lysates was carried out as described (St. Geme *et al.*, 1991).

Southern hybridization. Southern blotting was performed using high stringency conditions as previously described (St. Geme and Falkow, 1991).

15 **Microscopy.**

i. Light microscopy. Samples of epithelial cells with associated bacteria were stained with Giemsa stain and examined by light microscopy as described (St. Geme and Falkow, 1990).

ii. Transmission electron microscopy. For transmission electron microscopy, bacteria were incubated with epithelial cell monolayers for four hours and were then rinsed four times with
20 PBS, fixed with 2% glutaraldehyde/1% osmium tetroxide in 0.1 M sodium phosphate buffer pH 6.4 for two hours on ice, and stained with 0.25% aqueous uranyl acetate overnight. Samples were then dehydrated in graded ethanol solutions and embedded in polybed. Ultrathin sections (0.4 µm) were examined in a Phillips 201c electron microscope.

As shown in Figure 2, DB117(pN187) incubated with monolayers for four hours
25 demonstrated intimate interaction with the epithelial cell surface and was occasionally found to be intracellular. In a given thin section, invaded cells generally contained one or two intracellular organisms. Of note, intracellular bacteria were more common in sections prepared with strain N187, an observation consistent with results using the gentamicin assay. In contrast, examination of samples prepared with strain DB117 carrying cloning
30 vector alone (pGJB103) failed to reveal internalized bacteria (not shown).

Example 2 HAP Immunization

Bacterial Strains. NTHi strains N187 and P860295 were isolated from middle ear fluid of children with acute otitis media, while NTHi strain TN106 was isolated from a patient with
35 pneumonia. Strain N187 is the strain from which the *hap* gene was originally cloned (Sanders *et al.*, 1993, Infect. Immun. 61:3966-3975; St. Geme *et al.*, 1994, Mol. Microbiol. 14:217-233). Strain P860295 was obtained from Dr. Charles Brinton (University of Pittsburgh), and strain TN106 was obtained from Dr. Eric Hansen (University of Texas, Southwestern School of Medicine). Strain TN106.P2 is a derivative of TN106 that was
40 recovered after plating on medium containing 100 µg/ml of streptomycin, then inoculating

into the nasopharynx of a Balb/c mouse. Strains TN106.P2 and TN106 are indistinguishable in terms of morphology and growth characteristics. *H. influenzae* strain DB117 is a rec1 mutant of Rd, a capsule-deficient serotype d strain (Setlow *et al.*, 1968, J. Bacteriol. 95:546-558). DB117 contains a nonfunctional *hap* gene because of a spontaneous nonsense mutation at codon 710 and is nonadherent in assays with cultured epithelial cells (Fleischmann *et al.*, 1995. Rd. Science 269:496-512.).

H. influenzae strains were grown on chocolate agar supplemented with 1% IsoVitaléX, on brain heart infusion agar supplemented with hemin and NAD (BHI-XV agar), or in brain-heart infusion broth supplemented with hemin and NAD (BHIs), as described previously (St. Geme and Falkow, 1990, *supra*). These strains were stored at –80°C in BHI broth with 20% glycerol. *E. coli* strains were grown on Luria-Bertani (LB) agar or in LB broth and were stored at –80°C in LB broth with 50% glycerol. Antibiotic concentrations used to select for plasmids included 5µg/ml tetracycline in *H. influenzae* and 100 µg/ml ampicillin and 12.5 µg/ml tetracycline in *E. coli*. DNA ligations, restriction endonuclease digestions and gel electrophoresis were performed according to standard techniques (Sambrook *et al.*, 1989, *supra*). Plasmids were introduced into *E. coli* by electroporation (Dower *et al.*, 1988, *supra*). In *H. influenzae*, transformation was performed using the MIV method of Herriott *et al.* (Herriott *et al.*, 1970, *supra*).

Cloning and sequencing of *hap* from strains P860295 and TN106

The *hap* gene was cloned from strains P860295 and TN106 using the polymerase chain reaction (PCR) and primers corresponding to sequence flanking *hap* in strain N187. Reactions were performed with Expand polymerase (Roche Molecular Biochemicals) to enhance long range amplification and to minimize PCR-related errors. The 5' primer was based on sequence beginning approximately 500 base pairs upstream of *hap* (5'-TGCAGGATCCCCGCAGACTGGATTGTTG-3') (SEQ ID NO: 56), and the 3' primer corresponded to sequence beginning roughly 50 base pairs downstream of *hap* (5'-TGCAGGATCCGATCTGCCCCACCTTGTT-3')(SEQ ID NO: 57). To facilitate initial cloning, both the 5' and the 3' primers included a BamHI site. The amplified genes were cloned into BamHI-digested pUC19 and BglII-digested pGJB103.

Nucleotide sequencing was performed using an Applied Biosystems automated sequencer and the Big Dye Terminator Premix-20 kit (Applied Biosystems/Perkins Elmer). Double-stranded plasmid DNA was used as template, and sequencing was carried out along both strands. With strain TN106, clones from two separate PCR assays were sequenced, and the two sequences were identical. With strain P860295, a single clone was sequenced. The sequences for *hap*TN106 and *hap*P860295 have been submitted to GeneBank and are awaiting accession numbers.

The *hap*TN106 gene encodes a protein with 1392 amino acids, and the *hap*P860295 gene encodes a slightly larger protein with a total of 1436 amino acids. HapTN106 is 80% similar and 77% identical to HapN187, while HapP860295 is 85% similar and 83% identical to HapN187. Overall, the predicted amino acid sequences of Hap TN106 and Hap P860295 are 82% similar and 79% identical to each other.

As shown in Figure 11, alignment of the amino acid sequences of Hap TN106, HapP860295, and HapN187 revealed absolute identity through the first 47 amino acids, then significant divergence over the next 350 amino acids, and then marked similarity over the final 1000-1050 amino acids. Of note, the signal peptide and the sequence containing the active site serine residue (GDSGSPM) (SEQ ID NO: 58) are completely conserved in all three proteins. Similarly, the amino acids in the P1 (leucine), P3 (serine), and P4 (glutamine) positions of the primary autoproteolytic cleavage site between Haps and HapB are invariant.

Protein analysis and Western immunoblotting

Outer membrane proteins were isolated from *H. influenzae* on the basis of sarcosyl insolubility, as described previously (Carlone *et al.*, 1986, *supra*). Proteins released into the culture supernatant were precipitated using trichloroacetic acid, as described previously (St. Geme *et al.*, 1994, *Mol. Microbiol.* 14:217-233). Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 7-10% polyacrylamide gels, and Western blots were performed as detailed elsewhere (Laemmli, 1970, *supra*; Towbin, *et al.*, 1979, *Proc. Natl. Acad. Sci. USA* 76:4350-4354). Hap was detected using guinea pig antiserum GP74, which was raised against purified Haps and reacts with full-length Hap and Hap_s.

As shown in Figure 12, Western analysis of outer membrane proteins and secreted proteins from strain DB117/pHap(TN106) revealed full-length protein at ~155 kDa and Hap_s at ~110 kDa, virtually identical to control samples from strain DB117/pJS106 (HapN187). Examination of protein samples from strain DB117/pHap(P860295) revealed that full-length Hap migrated at ~160 kDa and that Haps migrated at ~115 kDa.

Adherence assays

Adherence assays were performed with Chang conjunctival epithelial cells (Wong-Kilbourne derivative, clone 1-5c-4 [human conjunctiva], as previously described (St. Geme, *et al.*, 1993, *supra*). Percent adherence was calculated by dividing the number of adherent colony-forming units per monolayer by the number of inoculated colony-forming units.

To assess adhesive activity, DB117/pHap(TN106) and DB117/pHap(P860295) were compared with DB117/pJS106 in adherence assays with Chang conjunctival epithelial cells. As shown in Figure 13, both Hap TN106 and HapP860295 promoted appreciable levels of adherence to these cells, similar to levels associated with HapN187. To extend this result, we examined whether antiserum raised against Haps purified from strain N187 could block adherence mediated by either HapP860295 or Hap TN106. As shown in Figure 13, in experiments with DB117/pHap(TN106), preincubation with a 1:500 dilution of guinea pig antiserum GP74 resulted in a nearly 50% decrease in adherence, and preincubation with a 1:100 dilution of GP74 resulted in a 70% decrease in adherence. Similarly, with DB117/pHap(P860295), a 1:500 dilution of GP74 blocked adherence by 40%, and a 1:100 dilution of GP74 blocked adherence by almost 80%.

Purification of NTHi P860295 Hap_s protein. To purify Hap_s from strain P860295, bacteria were grown in BHI broth for 18 hours at 35°C with aeration, i.e. to stationary phase. The bacterial cells were pelleted by centrifuging at 10,000 x g at 4°C and were discarded. Without being bound by theory, it is thought that the autoproteolysis of HAP results in

secretion of Hap_s. As such, the supernatant was collected and concentrated 20-fold using an Amicon stirred cell, then adjusted to 60% saturation with ammonium sulfate powder, incubated overnight at 4°C, and centrifuged at 17,000 x g for 1 hour at 4°C. The resulting precipitate was dissolved in 50 mM sodium phosphate buffer, pH 5.8, 1 mM EDTA, 50 mM NaCl and was dialyzed at 4°C against the same buffer (Buffer 1), then centrifuged at 100,000 x g for 1 hour at 4°C to remove insoluble material. A 10 ml bed volume CM sepharose (Pharmacia) column was equilibrated with Buffer 1, and 70 ml of the above soluble material was loaded onto the column at a flow rate of 5 ml/min. The column was washed with Buffer 1 until the OD280 reached baseline, and the flow through material was discarded. Next the column was washed with 3 column volumes of 50 mM sodium phosphate buffer, pH7.0, 1 mM EDTA, 50 mM NaCl. Hap_s was eluted with 50 mM sodium phosphate buffer, pH 8.0, 1 mM EDTA, 0.5 M NaCl. Fractions were examined by SDS-PAGE analysis, and fractions containing Haps were pooled. Hap_s from NTHi strain N187 was purified as previously described (Hendrixson, *et al.*, 1997, Mol. Microbiol. 26:505-518; Hendrixson and St. Geme, 1998, Mol. Cell 2:841-850). Strain P860295 Hap_s was purified from the native strain, while strain N187 Hap_s was purified from DB117 harboring pJS106 (encoding HapN187). Using this purification scheme, highly pure protein from both strain P860295 and strain N187 (Figure 14) was recovered. Amino terminal amino acid sequencing (described below) confirmed that purified protein was Hap_s.

Determination of N-terminal amino acid sequence To confirm the identity of purified Hap_s, protein was resolved by SDS-PAGE, then electrotransferred to a polyvinylidene membrane. After staining with Coomassie brilliant blue R-250, protein was excised from the membrane and submitted to Midwest Analytical, Inc. (St. Louis, MO). Amino-terminal sequence determination was performed by automated Edman degradation using a Perkin-Elmer Biosystems model 477A sequencing system.

Intranasal immunization of mice. Groups of ten, 6-week old, female Balb/c mice were immunized intranasally with Hap_s purified from either strain P860295 or strain N187. Hap_s was diluted in Dulbecco's PBS (D-PBS) to a final concentration of 5 or 15 µg/40 µl, with or without 0.1 µg CT-E29H (a mutant cholera toxin used as an adjuvant) (Tebbey, *et al.*, 2000, Vaccine 18:2723-34). Control mice received D-PBS alone or D-PBS with 0.1 µg CT-E29H, again in 40 µl volumes.

Prior to intranasal immunization, mice were anesthetized with an injectable mixture of ketamine (0.008 mls x body weight)/xylazene (0.007 mls x body weight), a mixture that maintains a state of anesthesia for 15-20 minutes. Immunizing preparations were delivered by pipette in a volume of 20 µl/nostril. The pipette was positioned so that the tip touched the opening of the nostril and liquid was drawn into the nasopharynx during breathing. Immediately following immunization, mice were placed in a supine position for a 3 to 5 minutes. Mice were immunized at weeks 0, 1, 3, and 5.

Intranasal challenge of mice. Either two or three weeks after the final immunization, animals were challenged intranasally with approximately 1 x 10⁶ CFU of strain TN106.P2. The TN106.P2 challenge strain was prepared for challenge by first inoculating three BBL Chocolate II agar plates from frozen stocks. Plates were incubated overnight at 37°C in 5% CO₂. Five ml of D-PBS was added to each plate and bacteria were resuspended with a

curved glass rod. Bacteria from all three plates were combined with an additional 10 ml of D-PBS and the suspension was poured over a D-PBS pre-wetted nylon wool column to remove clumps of bacteria and debris. The suspension was diluted with D-PBS to an OD₄₉₀=0.33, which was shown to equal $\sim 1.0 \times 10^8$ CFU/ml. This suspension was used for challenge. Mice were anesthetized as described for immunization, and 5 μ l of bacteria were administered in each nostril. Twenty minutes after the challenge began, an aliquot of the bacterial suspension was diluted in D-PBS and plated onto BHI-XV agar to determine the actual inoculum. Three days after challenge, nasal tissue was harvested, weighed, homogenized and plated on BHI-XV plates containing 100 μ g/ml streptomycin. Following incubation of plates overnight, colonies were counted, and CFU/g of nasal tissue were determined. Statistical differences among groups were analyzed using the Student t-test (JMP Software v3.2.1)

Measurement of serum antibody responses by ELISA To quantitate serum antibody responses against Hap_s, purified Hap_s was diluted and then blocked with 1% bovine serum albumin (BSA)/PBS at 37°C for 2 hours. Mouse sera were diluted in 1% BSA/PBS/0.05% Tween 20 (diluent buffer) and were transferred to coated and blocked plates. After incubation at 37°C overnight, plates were washed and then incubated with a 1:15,000 dilution of biotinylated rabbit anti-mouse IgG at 37°C for 2 hours. Next plates were washed again and then incubated with a 1:10,000 dilution of streptavidin-HRP (Zymed) at room temperature for 30 minutes. Finally, plates were washed and incubated with ABTS peroxidase substrate (Kirkegaard and Perry Laboratories) at room temperature for 20 minutes. Reactions were stopped with 1% SDS, and absorbance of ABTS was measured at 405 nm. ELISA endpoints were defined as the highest dilution of sera giving an OD₄₀₅ of >0.1. Control wells containing all reagents except for mouse sera had baseline OD₄₀₅ values of <0.05.

Serum antibody responses. Animals immunized IN will primarily produce a secretory immune response. Addition of CT-E29H increases the secretory immune response and also helps induce a serum antibody response. The volumes of the immunogens used in this experiment (40 μ l) probably resulted in some of the material being aspirated into the mice's lungs, further increasing the immune response in the serum. The anti-Hap_s ELISA titers of the sera obtained from immunized mice are shown in Table 2. The titers are somewhat lower than those usually seen with parenteral immunization since animals were immunized via the IN route. Significant increases in anti-Hap_s titers were seen in the sera. The responses increase in a dose dependent manner and are augmented approximately 3-fold by addition of 0.1 μ g of CT-E29H. This augmentation occurs at both dosage levels. No anti-Hap_s titers are seen in any of the control groups. Secretory IgA titers were not obtained from nasopharyngeal secretions.

TABLE 2

Systemic humoral immune response in Balb/c mice after intranasal vaccination with Hap _s admixed with or without CT-E29H				
Vaccine	Route (40 μ l)	Dose (μ g)	Adjuvant	Anti-Hap _s ELISA (IgG)
HAP	IN	5	–	1,604

	HAP	IN	15	–	5,204
	HAP	IN	5	CT-E29H	4,653
	HAP	IN	15	CT-E29H	15,111
	–	IN	–	CT-E29H	<500
5	1xPBS	IN	–	–	<500
	Formalin Fixed TN106.P2	IN		–	<500
6-week old female Balb/c mice were vaccinated week 0,1,3, & 5. Week 7 Sera - no antibody titers were detected in earlier bleeds.					

10 **Effect of Hap_s immunization on nasopharyngeal colonization.** IN immunization with purified
native Hap_s protein from NTHi strain P860295 significantly reduced the nasal colonization of NGH
strain TN106.P2 as shown in Figure 15. The reductions in recovered NTHi per gram of nasal
tissue ranged from 1.5 to 2.5 logs when animals were challenged 2 weeks after the last
immunization to approximately 1.5 logs when the animals were challenged 3 weeks after the
15 last immunization. all of the differences observed in groups immunized with Hap_s, whether with
or without CT-E29H, were significant as measured by the Student's T-test. The lowest level of
colonization for all groups was in the 5 µg Hap_s dose mixed with 0.1 µg CT-E29H. These results
indicate that IN immunization with Hap_s mixed CT-E29H given enhanced immune responses
as compared to IN immunization with Hap_s alone, and that the antibodies elicited by Hap_s are
20 biologically active against a heterologous NTHi challenge.

Having described the preferred embodiments of the present invention it will appear to those of
ordinary skill in the art that various modifications may be made to the disclosed embodiments,
and that such modifications are intended to be within the scope of the present invention.